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EVIDENCE FOR A SPONTANEOUS OVARIAN CYCLE IN FISH OF THE GENUS *XIPHOPHORUS* [1]

MICHAEL J. SICILIANO

Department of Biology, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

The reproductive cycle of fish of the genus *Xiphophorus*, as well as certain other poeciliid fishes, has been described by Bailey (1933) and Turner (1937). These viviparous Teleosts were found capable of producing broods throughout the year. After insemination by a male, sperm usually continue to survive in the folds of the female ovarian tissue and as many as 8 successive broods can be produced by one insemination (VanOordt, 1928). Vallowe (1953) calculated a mean interval of 33.5 days between broods of *Xiphophorus maculatus* (platyfish). The calculation of the number of days from insemination to the dropping of the first or primary brood depended on his ability to observe successful copulations. He determined the mean number of days to the dropping of 19 primary broods to be 41.7 and suggested that some factor, associated with the ovary, was involved in delaying the birth of primary broods. Similar results have been reported for the guppy *Poecilia reticulatus*, by Rosenthal (1952) who attributed the wide variation in time for the production of primary broods to an "ill-defined" estrus cycle.

The nature of this cycle should be distinguishable by utilizing artificial insemination to precisely fix the moment of insemination and to produce large numbers of primary broods. By plotting the length of time required to produce primary broods against the time to produce subsequent broods (as suggested by J. W. Atz, American Museum of Natural History, personal communication), the nature of the ovarian cycle operating in these fish could be more adequately analyzed. As part of a study in which it was necessary to produce large numbers of hybrids between fish of the genus *Xiphophorus*, such a procedure was carried out and is reported here.

Materials and Methods

All fish were the descendants of fish obtained from the Fish Genetics Laboratory of the New York Zoological Society in 1963. F_1, F_2 and backcross hybrids were produced between descendants of the swordtail, *Xiphophorus helleri strigatus* (Regan) originally collected from the Rio Sarabia, and the platyfish, *X. maculatus* (Guenther) strain 163A originally collected from the Rio Jamapa. Most matings were performed using the artificial insemination technique described by Clark

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(1950). Some F_2 and backcross hybrids were produced naturally. Inseminations were performed randomly with respect to cross type and season of the year, over a 3-year period. Female fish artificially inseminated were in their second year of life and had been kept isolated from males since maturity.

Each artificially inseminated female fish was kept in a 5-gallon aquarium filled with conditioned tap water which contained an abundance of the filamentous alga,

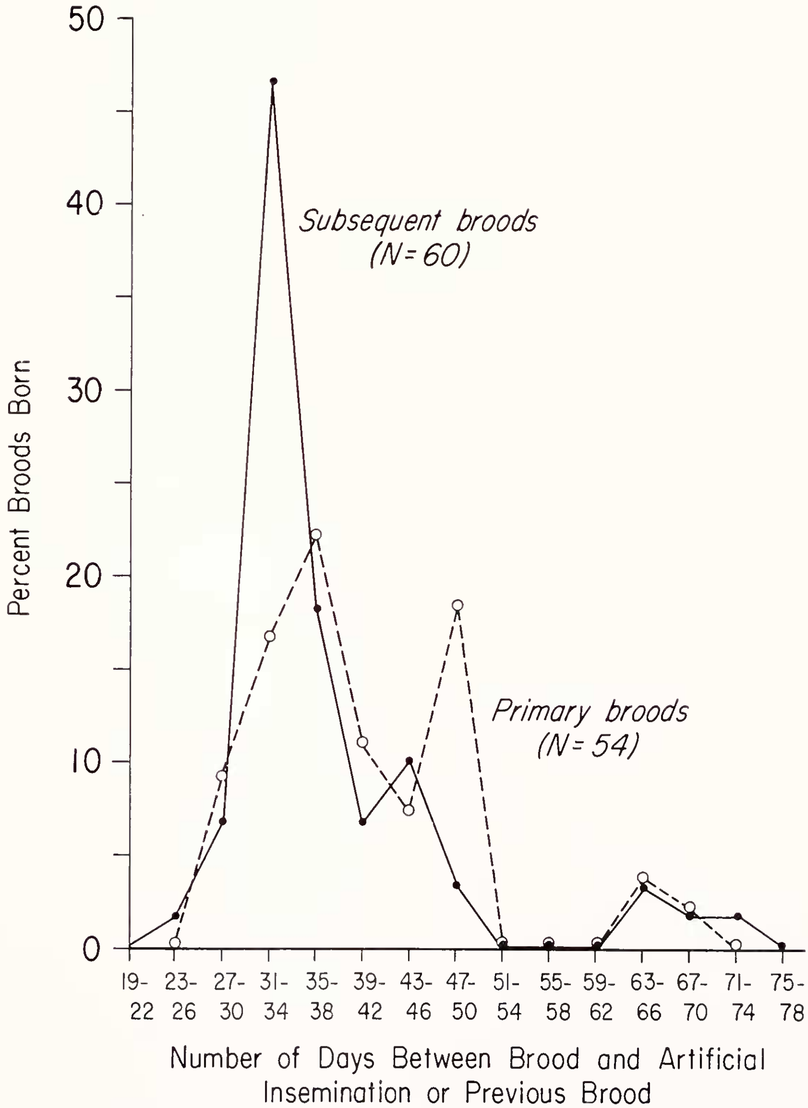

FIGURE 1. Percentage of primary broods born in 4-day intervals from 19 to 78 days following artificial insemination and subsequent broods born during the same intervals following the birth of previous broods.

Nitella. The water was under constant aeration and its temperature was maintained at 24° C ± 3°. No attempt was made to artificially control light. After birth of a brood (primary brood), the parent was immediately removed to another tank to await the birth of subsequent broods. The date each brood was dropped was recorded and the number of days between insemination and each primary brood was calculated. The date of each subsequent brood was recorded and the number of days between each brood was also calculated and compared to the number of days between insemination and each primary brood. Subsequent broods resulting from some natural matings were included in the data used to calculate the number of days between broods.

The percentage of primary broods dropped in 4-day intervals from 23 to 74 days after artificial insemination was scored and compared with the percentage of subsequent broods dropped during the same intervals following the birth of a previous brood. Any differences in the percentage of primary *versus* subsequent broods during such a particular time period were tested for statistical significance by making use of the binomial probability distribution (Sokal and Rohlf, 1969, pages 65–98). All such calculations were performed on a high-speed computer.

The mean number of days to the dropping of broods in various categories was compared and analyzed for statistical significance by the Student's t test.

Results

A total of 54 primary and 60 subsequent F_1, F_2, backcross to the swordtail (XXP-BC), and backcross to the platy (XPP-BC) broods were produced. The number of days between artificial inseminations and the dropping of primary broods, and the number of days between subsequent broods and previous broods, under the conditions of this laboratory, are compared in Figure 1. In addition to these broods, single primary broods were also dropped on the 79th, 91st, 106th, 155th, and 181st days following artificial inseminations.

The highest number of subsequent broods was dropped from 31 to 34 days after a previous brood (subsequent peak period). A peak number of primary broods was dropped from 35 to 38 days after artificial insemination (first primary peak) and again from 47 to 50 days after artificial insemination (second primary peak). The percentage of primary broods born during the second primary peak time period is significantly higher than the percentage of subsequent broods born during the equivalent time period ($P < 0.01$). The same is true for the percentage of subsequent broods dropped during the subsequent peak period with respect to the percentage of primary broods born during the equivalent time period. In no other time categories does the percentage of primary *versus* subsequent broods differ significantly.

Almost all broods were dropped from 23 to 50 days following artificial insemination or the birth of a previous brood. The mean number of days for all broods born within this time period was 36.6 ± 0.6. The mean for primary broods was 38.3 ± 1.0 days while the mean for subsequent broods was 35.1 ± 0.7 days. The difference between these means is highly significant ($P < 0.01$).

Primary broods dropped during the later portion of the 23- to 50-day time period (37–50 days after artificial insemination) are listed, along with other data relative to the inseminations, in Table I. Similar information is contained in

Table II for primary broods dropped during the early portion of the 23 to 50 day time period (23 to 36 days after artificial insemination).

The mean number of days to the dropping of subsequent broods from females who dropped their primary broods during the later portion (37–50 days after artificial insemination) of the 23- to 50-day time period (34.8 ± 1.2) was not statistically significant when compared to females who dropped their primary broods during the early portion (23 to 36 days after artificial insemination) of the 23- to 50-day time period (35.4 ± 0.8).

TABLE I

Primary broods born from 37 to 50 days following artificial insemination

Insemination number	# Days to birth of primary brood (x_1)	# Days to birth of subsequent broods (x_2)	Female parent	Type of hybrids produced	Month/day of insemination
22	45	—	Platy	F_1	2/28
30	49	31, 31	Swordtail	XXP-BC	6/7
31	49	—	Swordtail	XXP-BC	6/7
32	49	—	Swordtail	XXP-BC	6/7
33	49	32, 31	Swordtail	XXP-BC	6/7
34	47	35, 48, 40	F_1	F_2	2/13
36	47	31, 30	F_1	F_2	2/13
41	47	—	Swordtail	XXP-BC	2/1
43	49	—	Swordtail	XXP-BC	6/24
58	40	—	Platy	F_1	11/17
61	41	33, 32	F_1	XPP-BC	11/17
64	37	—	F_1	XPP-BC	4/11
66	43	37, 41	Swordtail	XXP-BC	11/17
67	45	—	Swordtail	XXP-BC	11/18
68	40	33	Platy	XPP-BC	11/18
82	49	35, 46	F_1	XXP-BC	11/19
86	50	31	F_1	F_2	11/25
95	38	33	Platy	F_1	4/4
97	41	—	Platy	F_1	4/10
113	40	30, 35	F_1	XPP-BC	8/20
114	40	—	F_1	XPP-BC	8/20
117	43	—	F_1	XPP-BC	8/20
Means	$\bar{x}_1 = 44.5 \pm 0.9$	$\bar{x}_2 = 34.8 \pm 1.2$			
	$t = 6.6, P < 0.01$				

DISCUSSION

The data are consistent with the observations made by Vallowe (1953) that the mean time to the first or primary brood is longer than the mean interval between subsequent broods in fish of the genus *Xiphophorus*. However, by using artificial insemination to precisely fix the moment of sperm introduction in a large number of broods and by plotting the data modally, a general lag to the production of primary broods is not observed. Primary broods were dropped in two peak periods (1st and 2nd primary peaks). The time of the 1st primary peak roughly corresponds to the peak period in which subsequent broods were dropped. Therefore, the major reason for the higher mean time for the dropping of primary broods, must have been due to the broods dropped in and around the second primary peak. The fact that the percentage of primary broods dropped within this period was significantly higher than the percentage of subsequent broods dropped from 47–50 days after the birth of a previous brood indicates that the second primary peak is present for reasons other than random variation of the gestation time of indivdual broods in the two groups.

It could be argued that there may be a nonrandom difference in the gestation time of the fish that dropped primary broods in and around the second primary peak as opposed to the fish which dropped primary as well as subsequent broods earlier. This could be considered a manifestation of the time of year these inseminations were carried out or the type of hybrids produced in the brood. However, that this is not the case is indicated by the data in Tables I and II. Primary broods dropped during the later portion of the 23- to 50-day period (37–50 days after artificial insemination) present the same spectrum of F_1, F_2, XXP-BC, and XPP-BC hybrids, and were inseminated at equivalent times of the year as those dropped during the earlier portion. That these fish do not have abnormally long gestation periods is indicated by the fact that the length of time to the dropping of their subsequent broods is equivalent to the interval

TABLE II

Primary broods born from 28 to 36 days following artificial insemination

Insemination number	# Days to birth of primary brood (x_1)	# Days to birth of subsequent broods (x_2)	Female parent	Type of hybrids produced	Month/day of insemination
1	30	—	Swordtail	XXP-BC	5/6
4	28	—	Platy	F_1	10/20
6	31	—	Platy	F_1	10/20
8	34	36	Swordtail	F_1	10/27
12	35	34	Platy	F_1	1/13
13	32	37	Swordtail	F_1	1/13
25	35	—	Swordtail	F_1	3/28
29	35	30	Swordtail	F_1	5/16
39	35	—	F_1	XPP-BC	2/1
40	35	33, 35, 35, 41	F_1	XPP-BC	2/1
45	28	34	Swordtail	XXP-BC	6/24
56	35	45	Swordtail	F_1	6/25
73	36	—	Swordtail	XXP-BC	11/18
75	31	33, 43, 31	F_1	XXP-BC	11/19
76	31	—	F_1	XXP-BC	11/19
77	36	—	F_1	XXP-BC	11/19
78	31	33, 43, 31	F_1	XXP-BC	11/19
80	31	38, 32, 37	F_1	XXP-BC	11/19
104	35	—	Platy	XPP-BC	6/19
116	33	—	F_1	XPP-BC	8/20
121	30	32, 35	Swordtail	F_1	8/20
123	35	33, 37	Swordtail	F_1	8/20
128	28	—	Platy	F_1	10/15
129	34	32	Platy	F_1	8/29
Means	$\bar{x}_1 = 32.7 \pm 0.6$	$\bar{x}_2 = 35.4 \pm 0.8$			
	$t = 8.7$, $P < 0.01$				

between broods of fish whose primary broods were dropped during the earlier portion of the 23- to 50-day period. Primary broods dropped in and around the second primary peak must have been the results of fertilizations which were delayed independently of time of year and cross type. Therefore, in some inseminations (indeed most) fertilization was quick and in others, delayed.

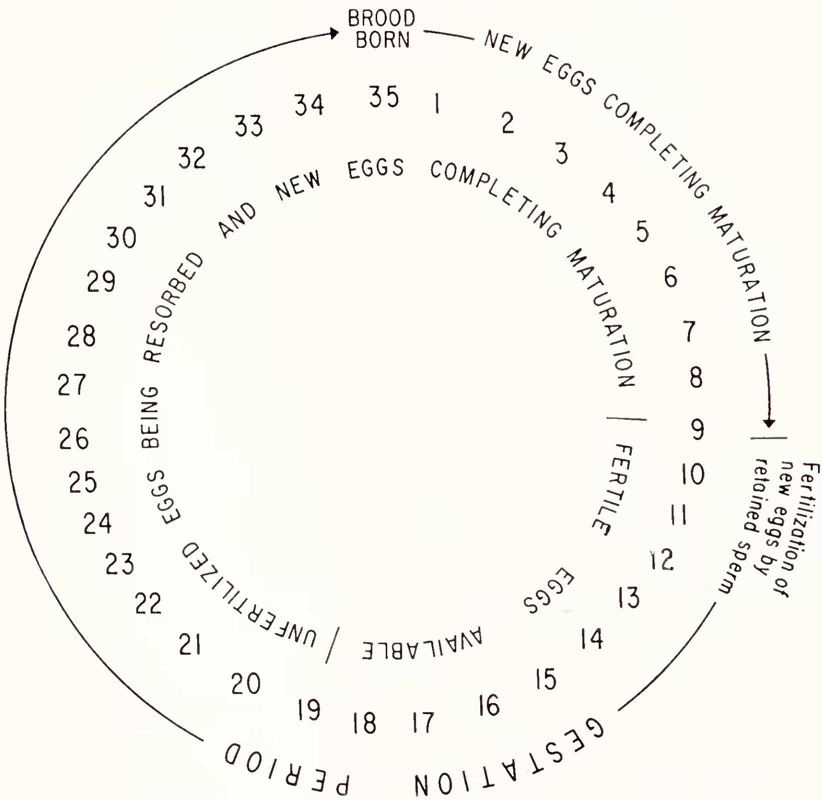

FIGURE 2. Model spontaneous ovarian cycle for fish of the genus *Xiphophorus*. The days for a typical cycle in the fish used in this laboratory are indicated by the numbers. The events which take place in an impregnated fish are indicated peripheral and adjacent to the days of the cycle in which they occur. The events which take place in a virgin fish are indicated inside and adjacent to the appropriate days of the cycle in which they occur.

The presence of a single peak period (conforming to the mean number of days after dropping of previous brood) for the dropping of subsequent broods is in conformity with brood interval data reported by numerous investigators. The mean number of days to the dropping of subsequent broods reported here (35.1 ± 0.7) is higher than that reported in the past and is most likely a reflection of the relatively constant cool temperatures maintained.

The finding that primary broods are born in two peak periods, an earlier one conforming to the subsequent peak period and a later one from 47–50 days after artificial insemination, is not inconsistent with the results of Vallowe (1953), Rosenthal (1952) or Clark (1950). Measuring the time from observed copulation, cohabitation with males, and artificial insemination, respectively, each of these investigators reported females dropping primary broods from a time approximating the interval between broods up to about 50 days after the initiation of the experiment. The relatively few number of reported cases and the relatively imprecise method of determining the moment of insemination (in Rosenthal's experiments) does not allow an analysis of peaks. It was suggested (Rosenthal, 1952) that the wide spread in time for the production of primary broods was the result of an ill-defined estrus cycle—ova mature and in the absence of fertilization are periodically resorbed.

An interesting result is seen in Clark's data. Although she only reported 9 successful artificial inseminations, in one of them a brood was produced in only 22 days. The earliest subsequent brood obtained was dropped by the same fish in 26 days, followed by another in 27 days. In the present experiment, conducted under highly controlled temperature conditions, the earliest brood was a subsequent, not a primary. However, as indicated in Table II, the mean time to the dropping of primary broods born in less than 37 days after artificial insemination was significantly less than the mean interval between broods in those fish. Ten of 13 of these fish which dropped subsequent broods produced their primary broods in less time than at least one of their subsequent broods. These results are interpreted as indicating that in many virgin fish, fertilization and the beginning of the gestation take place sooner after artificial insemination than they do after the birth of a previous brood.

These observations can be explained by the presence of a spontaneous ovarian cycle which is present in all mature female fish—virgin or inseminated. The length of this cycle, which is temperature dependent, can be measured by the interval between broods. Under the conditions of this laboratory the typical cycle was 35 days (Fig. 2). One may consider a female who has dropped a brood, to have dropped it on the 35th day of the cycle. The next day would mark day "1" of the next cycle. Since Tavolga (1949) calculated the completion of egg maturation to take 6.8 days following the birth of a brood when the brood interval was 28 days, in the present study the egg maturation period is considered to be 8 days followed by a 27-day gestation period. A fish may therefore be considered to contain mostly fertile eggs starting on the 8th day of the cycle. (Turner 1937, pointed out that all eggs, and consequently all embryos, are not at the same maturation stage at the same time. However, as Tavolga noted in 1949, they all are similarly developed at birth because of the slowness of the last embryological steps.) Eggs capable of being fertilized would be present in the ovary for the next 11 days through day 19 of the cycle (explained below). If not fertilized, the eggs would be resorbed followed by replacement with new eggs. These processes would consume 24 days lasting through the remaining 16 days of the cycle and into the first 8 days of the next cycle. It is suggested that this 35-day cycle is repeated during the mature period of a female fish whether or not it is ever fertilized.

Such a model spontaneous ovarian cycle explains the production of two peak periods of brood birth following artificial insemination of virgin fish. Primary broods born during the first half of the 23- to 50-day period following artificial insemination would theoretically conform to inseminations taking place during the first 19 days of the female's cycle. Many of these broods were dropped in less time than subsequent broods from the same female. Such a situation would be accounted for by inseminations taking place from day 8 of the cycle to day 19 (assuming most eggs were mature and available for fertilization).

Primary broods born during the latter half of the 23- to 50-day period would conform to inseminations taking place from the 20th to 35th day of the cycle. Sperm introduced at this point in the cycle would have no fertile eggs with which to combine. This sperm would be stored and be available to fertilize eggs, some of which would be ready on the 8th day of the next cycle. Such broods would be born at a minimum of 36 days after insemination (inseminations on the 34th day of the cycle) to a maximum of 50 days after insemination (inseminations on the 20th day of the cycle). Primary broods should not take longer than 50 days after artificial insemination since sperm introduced before the 20th day of the cycle should be in time to fertlize good eggs produced earlier in that cycle. The fact that 3 primary and 4 subsequent broods were seen from 63 to 74 days after artificial insemination or birth of a previous brood may be interpreted as broods which were missed or skipped a cycle. Primary broods dropped on the 79th, 91st, 106th, 155th and 181st days following artificial insemination were very likely from late maturing females.

A basic feature of this model is that fertile eggs were available for fertilization for only approximately 11 days (day 9 through 19 of the cycle). Why 11 days? If less than 11 days, 6 for instance, then fertile eggs would not be available for fertilization of the 16th, 17th, 18th and 19th days of the cycle. Sperm introduced at those points would have to wait 28, 27, 26 and 25 days, respectively, for fertile eggs to become available in the next cycle. These delays plus the 27 day gestation period would mean that broods in those cases would not be dropped until the 55th, 54th, 53rd and 52nd days following artificial insemination. Since no primary broods were born from 51 to 62 days following artificial insemination (Fig. 1), eggs capable of being fertilized must have been present through the 19th day of the cycle and therefore at least 11 days. If fertile eggs were present for more than 11 days, 16 for instance, they would have been available for fertilization on the 20th, 21st, 22nd, 23rd and 24th days of the cycle. This would mean that the earliest day of the cycle in which fertile eggs were not available would have been day 25. Sperm introduced on that day would have to wait only 19 days for fertile eggs to become available. Considering the 27 day gestation period, the maximum time for primary broods to be dropped following artificial insemination would have been 46 days. However, fully 18.5% of the primary broods were born from 47–50 days following artificial insemination (Fig. 1). Therefore, these broods appear to be the result of inseminations from the 20th to 24th days of the cycle during which time fertile eggs were not available.

An alternate interpretation could also be applied to these data. Perhaps a female fish which had not recently reproduced, possessed eggs which were not capable of being fertilized. Inseminations would then stimulate the resorption of these eggs and production of a new set. Such activity would account for a second primary peak independent on a spontaneous cycle. The first primary peak would be the result of inseminations into fish in which the eggs had not yet become overripe.

While further work is necessary to resolve these two interpretations, it is felt that the following observations speak in favor of the spontaneous cycle in fish of this genus: (1) Tavolga (1949) has noted that unfertilized degenerating eggs are of frequent occurrence, particularly in *virgin* females. (2) As pointed out by Turner (1937) several species in this family display superfoetation (more than one brood of embryos of different sizes in the ovary at the same time). Such a phenomenon is indicative of the fact that a spontaneous ovarian cycle is not strange to the family *Poeciliidae*.

Summary

1. The nature of the ovarian cycle in fish of the genus *Xiphophorus* was studied by using artificial insemination to precisely fix the moment of sperm introduction and by comparing the time to produce primary broods with subsequent broods.

2. The mean time to the production of primary broods was significantly longer than the time to produce subsequent broods. However, there was not a general lag in the production of primary broods but instead they were seen to be born in two peak periods—an earlier one conforming to the single subsequent brood peak, and a later one.

3. The presence of two primary peaks, the fact that the mean number of days to the production of those broods clustered around the first peak was significantly less than that of their subsequent broods, and the lack of the birth of any broods in the time periods immediately following the second peak suggests the presence of a spontaneous ovarian cycle in these fish.

4. Based on the time factors observed in this laboratory, a model cycle was constructed indicating its length (35 days), gestation period (27 days), and length of time fertile eggs were available in the ovary (11 days).

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